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Photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 stimulates wound healing in healing-impaired *db*/*db* mice

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Abstract

Application of ultraviolet light (UV-) irradiation to a photocrosslinkable chitosan (Az-CH-LA) aqueous solution including fibroblast growth factor-2 (FGF-2) resulted within 30 s in an insoluble, flexible hydrogel. About 20% of the FGF-2 molecules were released from the FGF-2-incorporated chitosan hydrogel into phosphate buffered saline (PBS) within 1 day, after which no further significant release occurred under in vitro non-degradation conditions of the hydrogel. The FGF-2 molecules retained in the chitosan hydrogel remained biologically active, and were released from the chitosan hydrogel upon the in vivo biodegradation of the hydrogel. In order to evaluate its accelerating effect on wound healing, full thickness skin incisions were made on the back of healing-impaired diabetic (*db*/*db*) mice and their normal (*db*/+) littermates. Application of the chitosan hydrogel significantly induced wound contraction and accelerated wound closure in both *db*/*db* and *db*/+ mice. However, the addition of FGF-2 in the chitosan hydrogel further accelerated wound closure in *db*/*db* mice, although not in *db*/+ mice. Histological examination also has demonstrated an advanced granulation tissue formation, capillary formation and epithelialization in wounds treated with FGF-2-incorporated chitosan hydrogels in *db*/*db* mice.

Keywords: Chitosan; FGF-2; Photocrosslinking; Diabetic ulcer; Wound healing

1. Introduction

The healing of a wound proceeds in three overlapping phases, namely (i) inflammation, (ii) granulation tissue formation, and (iii) matrix formation and remodeling [1]. This sequential process requires the interaction of cells in the dermis and epidermis, as well as the activity of chemical mediators released from inflammatory cells, fibroblasts and keratinocytes. The proliferation of mesenchymal cells and capillaries, as well as the influx of macrophages composing granulation tissue serve to replace the lost dermis and to provide substrates and inducers for re-epithelialization [1,2]. It is expected that application of growth factors that induce fibroblast and/or endothelial cell proliferation to healing-impaired wounds might increase the rate and degree of granulation tissue formation and thus stimulate wound repair.

It has been reported that several growth factors function in the process of wound healing. Especially, FGF-2 has many biological activities that stimulate the proliferation of fibroblast and capillary endothelial cells, thus promoting angiogenesis and wound repair [3–6]. However, the application of FGF-2 in healing-impaired wound treatments has not always been successful in vivo. One of the reasons for the difficulty is the high diffusibility and the very short half-life time of FGF-2 in vivo to retain its biological activity [7]. Therefore, it is required to stabilize and localize the in vivo activity in order to apply FGF-2 in wound repair and tissue regeneration.

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We have previously reported the preparation and characterization of a novel photocrosslinkable chitosan [8]. The material is a viscous solution and is easily crosslinked upon ultraviolet light (UV-) irradiation, resulting in an insoluble hydrogel. The chitosan hydrogel is a strong tissue-adhesive and, when compared with a fibrin glue, is more effective in sealing air leakages from pinholes on isolated small intestines and aorta, as well as from incisions on isolated trachea [9]. Furthermore, the chitosan hydrogel has been found to induce wound contraction and healing [10,11]. The purpose of the present study has been to evaluate the chitosan hydrogel as a carrier material for controlled release of FGF-2 and to assess possibilities of the FGF-2-incorporated chitosan hydrogel as a dressing for wound occlusion and healing acceleration in healing-impaired *db*/*db* mice [2,12]. The healing properties of the FGF-2-incorporated chitosan hydrogel-treated wound have been evaluated using a healing impaired wound model with full thickness-skin incisions and histological examinations.

2. Materials and methods

2.1. Preparation of photocrosslinkable chitosan molecules (Az-CH-LA)

Photocrosslinkable chitosan molecules (Az-CH-LA) have been prepared as previously reported [8]. The chitosan used in this study had a molecular weight of 800–1000 kDa with a deacetylation ratio of 0.8 (Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan). Azide (*p*-azidebenzoic acid) and lactose (lactobionic acid) moieties have been introduced through a condensation reaction with amino groups. The introduction of lactose resulted in a water-soluble chitosan at neutral pH-values. It has been estimated that about 2.5% and 2.0% of the amino groups in the chitosan reacted with *p*-azide benzoic acid and lactobionic acid, respectively [8]. A viscous Az-CH-LA aqueous solution (20–30 mg/ml) has been converted into an insoluble hydrogel within 10 s upon UV-irradiation at a lamp distance of 2 cm (UV-irradiation System, Spot Cure ML-251C/A with a guide fiber unit (SF-101BQ) and 250 W lamp (240–380 nm), Usio Electrics Co., Ltd., Tokyo, Japan) through crosslinking of the azide and amino groups of the Az-CH-LA molecules.

2.2. Determination of the FGF-2 release from a chitosan hydrogel

The release of FGF-2 from a chitosan hydrogel has been carried out in vitro in a phosphate buffered saline (PBS) solution. To prepare a FGF-2-incorporated chitosan hydrogel, 0.5 ml of 15 μg human recombinant FGF-2 (PeproTech EC Ltd., London, UK) in PBS was mixed into 1 ml of 30 mg/ml Az-CH-LA aqueous solution (finally 20 mg/ml Az-CH-LA solution) with vortex. The obtained Az-CH-LA solution (50 μl) containing FGF-2 was then spotted at the center of a well in a 24-multiwell tissue culture plate and converted into an insoluble hydrogel upon UV-irradiation for 10 s. The hydrogel was rinsed twice with PBS and gently shaken in 1 ml PBS on a rotary shaker at room temperature for indicated time periods. The PBS solution was changed every day. The concentration of FGF-2 in the PBS washes was determined using an ELISA assay [13].

2.3. Effect of FGF-2-incorporated chitosan hydrogel on the HUVEC growth

Human umbilical vein endothelial cells (HUVECs; Takara Biochemical Corp., Ohtsu, Japan) were cultured in medium-199 (Life Technologies Oriental Corp., Tokyo, Japan) supplemented with 10 wt% heat-inactivated fetal bovine serum (FBS), antibiotics (100U/ml penicillin G and 100 μg/ml streptomycin). The cells used in this work were all between the fourth and eighth cell cycle passage.

FGF-2 (15 μg) in 1 ml of PBS was mixed with 2 ml of Az-CH-LA aqueous solution (30 mg/ml). Az-CH-LA solution(50 μl) containing 250 ng of FGF-2 was spotted at the center of a well of a 24-multiwell culture plate and converted into an insoluble hydrogel upon UV-irradiation of 10 s. The photocrosslinked chitosan hydrogels containing the FGF-2 were washed with 1 ml PBS during the indicated days on a rotary shaker at room temperature. The PBS solutions were changed daily. HUVECs were then plated into the well in the presence of the washed chitosan hydrogel containing FGF-2 at an initial density of 15,000 cells/well in medium-199 supplemented with 10 wt% heat-inactivated FBS and antibiotics without addition of growth factor and incubated for a period of 3 days.

In order to examine the effect of partial degradation of FGF-2-incorporated chitosan hydrogels by chitinase (chitinase-RS; Seikagaku Corp., Tokyo, Japan) and chitosanase (chitosanase-RD; Seikagaku Corp.), HUVECs were plated into the well in the presence of a 7 days-washed FGF-2-incorporated chitosan hydrogel. The cells were incubated in the medium-199 supplemented 10% heat-inactivated FBS and antibiotics without growth factor as described above, but also contained chitinase (1 mg/ml) and chitosanase (2 mg/ml). The chitinase and chitosanase in the medium did not affect HUVEC growth, but did partially degrade the chitosan hydrogel. After 3 days incubation of the chitosan hydrogel in the medium including the chitinase and chitosanase, many tears in the hydrogel, as well as small fragments of the hydrogel, in the medium were observed, indicating partial degradation of the hydrogel.

2.4. Estimation of in vivo degradation of trypan blue-incorporated chitosan hydrogels

In vivo degradation of a chitosan hydrogel has been examined by determining the decrease of trypan blue in an implanted trypan blue-incorporated hydrogel. To prepare a trypan blue-incorporated chitosan hydrogel, 1 mg trypan blue (acidic dye; Sigma-Aldrich Japan Corp., Tokyo, Japan) in 1 ml PBS was mixed into 2 ml of 30 mg/ml Az-CH-LA aqueous solution with vortex. Since trypan blue was not released from a trypan blue-incorporated chitosan hydrogel in vitro, any decrease in trypan blue concentration of the hydrogel in vivo should be identical to the biodegradation of the hydrogel. The chitosan hydrogel formed from 100 μl of the trypan blue-containing Az-CH-LA (20 mg/ml) aqueous solution was carefully implanted into the right and left side of the back subcuits of mice (each weighing around 30 g, C57BL/6, female, 10-week-old, Crea Japan Inc., Tokyo, Japan) 2 cm apart from the tail root. The mice were sacrificed after 1, 2, 4, 7, 10, and 14 days post hydrogel implantation and subsequently the implanted hydrogels were removed. The blood was rinsed with PBS and tissue attached to the implants was removed. The obtained hydrogel was then treated with 0.5 ml of 100 mM $NaNO_2$ at pH 3.0 to degrade and centrifuged at 2000 rpm after a thorough vortex. The trypan blue concentration in the supernatant was determined by measuring the OD_{640}. Each experimental group was consisted of three mice.

2.5. Creation of skin wounds

Female mutant diabetic mice, C57BL/ksJ *db*/*db*, and their normal littermates (*db*/+) (CREA Japan Inc., Tokyo, Japan) were used in this study. All mice maintained a standard laboratory diet and water ad libitum, and were experimentally used when over 10 weeks of age. Prior to the experiments, mice were checked for urinary glucose and protein using reagent strips (Uro-Labstrix: Bayer Medical Ltd., Tokyo, Japan). And all the *db*/*db* and *db*/+ mice were diagnosed to be severely diabetic and normal, respectively. After being anesthetized with diethyl ether and completely cutting dorsal hair, full thickness-round wounds (about 100 mm^2) were prepared on the upper back of each mouse using a sharp pair of scissors and a scalpel. About 100 μl of 20 mg/ml of Az-CH-LA aqueous solution including either human recombinant FGF-2 (Pepro Tech EC Ltd., London, UK) or heparin (from porcine intestine, Scientific Protein Laboratories Corp., Waunakee, WIS, USA) was added onto the wound of each mouse and was then irradiated with UV light at a distance of 2 cm for 20 s. Similar full thickness-round wounds (about 100 mm^2) were also prepared as controls without any treatment. The changes in wound area of mice were measured using a slide caliper at day 2, 4, 8, and 16 (for *db*/*db* mice) and at day 2, 4, and 8 (for *db*/+mice) after initial wounding. The skin including the wound was removed from each mouse for histological examination. These animal experiments have been approved and carried out following the guidelines for animal experimentation at the National Defence Medical College, Tokorozawa, Saitama, Japan.

2.6. Histological examination

The removed skins including wound tissue were fixed in a 10% formaldehyde solution, embedded in paraffin and sectioned in 4 μm increments (Yamato Kohki Inc., Asaka, Saitama, Japan). The sections were made perpendicular to the anterior–posterior axis and perpendicular to the surface of the wound. The sections were positioned on a glass slide, and stained with hematoxylin-eosin (HE) reagent.

3. Results

3.1. Release of incorporated trypan blue and FGF-2 from chitosan hydrogels

Fig. 1A shows the release profiles of 2 dyes, trypan blue (an acidic molecule, MW 961 Da) and toluidine blue (a basic molecule, MW 306 Da), from chitosan

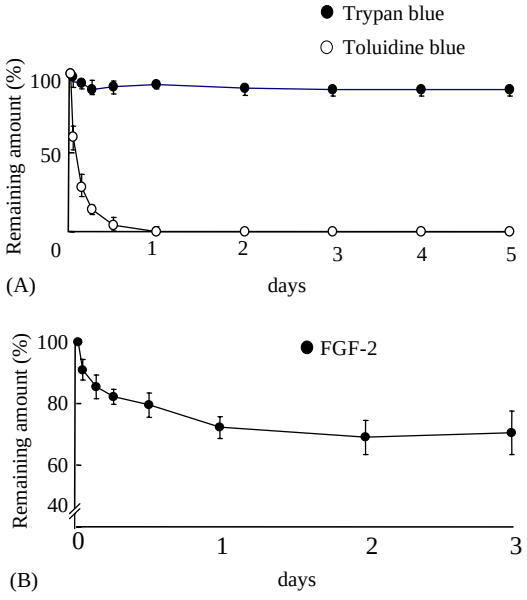


Fig. 1. Time course of remaining incorporated molecules in the chitosan hydrogel. The concentration of released trypan blue and toluidine blue (A), and FGF-2 (B) in the PBS wash was quantified, as described in Section 2. The amount of initially incorporated molecules in the chitosan hydrogel is defined as 100%. Each data point represents the mean ± SD of triplicate determinations.

hydrogels at room temperature in PBS. While almost all toluidine blue molecules were released from the hydrogel within 1 day, no release of trypan blue molecules was observed over a period of 5 days (Fig. 1A). The fast release of toluidine blue molecules can be ascribed to a simple diffusion in the hydrogel, since no electrostatic interaction exists between toluidine blue and chitosan molecules. On the contrary, trypan blue, an acidic molecule, electrostatically bound to the chitosan hydrogel, has not been found to be released from the gel under in vitro non-degradation conditions. Therefore, the trypan blue-incorporated chitosan hydrogel was used as indicator of in vivo degradation of the chitosan hydrogel.

Fig. 1B shows the release profile of FGF-2 from the chitosan hydrogel. Approximately 20% of the incorporated FGF-2 molecules were found to be released from the chitosan hydrogels within the first day, followed by no substantial further release after that. Initial small release of FGF-2 (about 20%) from chitosan hydrogels may be explained in terms of this molecular diffusion. Present FGF-2 immobilization conditions into the chitosan hydrogel were not sufficient to complete polyion complexation between FGF-2 molecules and the basic chitosan hydrogel, resulting in diffusion of uncomplexed FGF-2 initially.

3.2. Effect of FGF-2-incorporated chitosan hydrogels on HUVEC growth

FGF-2 is well known to be a stimulating factor for endothelial cell growth [4–6]. The FGF-2-incorporated chitosan hydrogel was found to be able to stimulate HUVEC growth, but washing of the FGF-2-incorporated chitosan hydrogels with PBS for longer than 3 days resulted in a loss of ability to stimulate HUVEC growth (Fig. 2). Washing of a FGF-2-incorporated chitosan hydrogel with PBS (changed daily) for 3 days appeared to result in a lowering of release of the FGF-2 molecules into the culture medium, identical to the result found in Fig. 1B.

In order to examine the stability of the FGF-2 in a chitosan hydrogel, HUVECs were cultured in the presence of 7 days-washed FGF-2-incorporated chitosan hydrogel and both chitinase and chitosanase. HUVECs grew normally in the culture medium containing the chitinase and chitosanase (data not shown). The enzymes in the culture medium did result in many tears in the chitosan hydrogel, as well as small fragments of the hydrogel in the medium after 3 days incubation (data not shown). Although 7 days-washed FGF-2-incorporated chitosan hydrogels entirely lost the ability to stimulate HUVEC growth, the stimulating activity on the HUVEC growth was almost recovered upon treating the growth factor-incorporated chitosan hydrogel with chitinase and chitosanase (Fig. 2). These results indicate

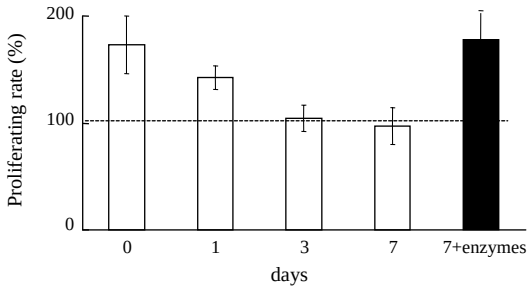


Fig. 2. Effect of FGF-2-incorporated chitosan hydrogel on the growth of HUVECs in vitro. FGF-2-incorporated chitosan hydrogels were washed with PBS for the indicated period of time and HUVEC cultured (white bars). After incubation for 3 days, the number of grown cells was measured, as described in Section 2. The black bar represents cells cultured in the presence of chitinase/chitosanase for 3 days after washing the FGF-2-incorporated chitosan hydrogel with PBS for 7 days. The cells incubated with a pre-washed chitosan hydrogel without FGF-2 have been defined as 100% (dotted line). The cell growth data were then calculated as a percentage, representing the mean ± SD of tetraplicated determinations.

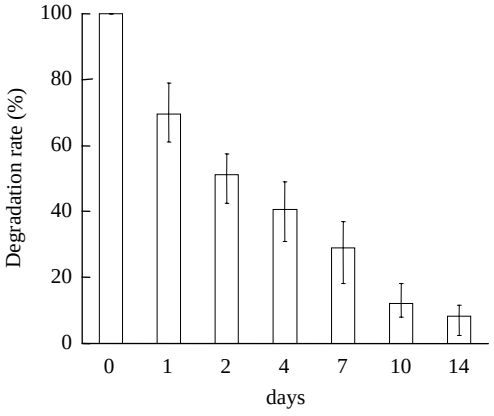


Fig. 3. Biodegradation of trypan blue-incorporated chitosan hydrogels. Biodegradation of trypan blue-incorporated chitosan hydrogels into the back subcuits of mice was examined, as described in Section 2. The amount of initially incorporated trypan blue has been defined as 100%. The data represent the mean ± SD of six determinations (three mice for each point).

that partial degradation of the FGF-2-incorporated chitosan hydrogels did occur in the presence of chitinase and chitosanase, and that growth factors within the chitosan hydrogel retained their biologically active form during a 7 days-period of hydrogel washing.

3.3. Estimation of in vivo degradation of trypan blue-incorporated chitosan hydrogel

In vivo degradation of a chitosan hydrogel (100 μl per mouse) was examined using a trypan blue-incorporated chitosan hydrogel. As found, the interacted trypan blue molecules with the chitosan hydrogel would not release without degradation of the chitosan hydrogel itself. The

dye in the trypan blue-incorporated chitosan hydrogel, implanted into the back of mice, decreased with implantation time (Fig. 3). About 70%, 50%, and 25% of the dye molecules were retained in the implanted chitosan hydrogel after day 1, 2, and 7 post-operation, respectively. However, many diffused blue fragments around an implanted site were observed after day 10 and 14. Thus, the implanted chitosan hydrogel was partially biodegraded in vivo in about 10–14 days when implantation.

3.4. In vivo wound healing experiments

In our wound healing mouse model, a single full thickness circular skin wound (about 100 mm^2) was made on the back of *db*/*db* and *db*/+mice. The rate of wound closure for each wound has been evaluated by determination of the unclosed wound area as a function of time. A chitosan hydrogel or FGF-2-incorporated chitosan hydrogel applied to the wound retained hydrated throughout the wound healing process. For the *db*/*db* mice treated with the FGF-2-incorporated chitosan hydrogel, healing was faster than without FGF-2-incorporation and about 80% wound closure could be achieved within 12 days (Fig. 4). In contrast with the FGF-2-incorporated chitosan hydrogel-treated wounds of *db*/*db* mice, control wounds healed more slowly and about 80% wound closure was achieved only after over 20 days (Fig. 4). Healing in only chitosan hydrogel-treated wounds of *db*/*db* were found to be intermediate (Fig. 4). More precisely, substantial wound contractions in both the chitosan hydrogel-treated wounds and FGF-2-incorporated chitosan hydrogel-treated wounds were observed in the first 2 days, namely

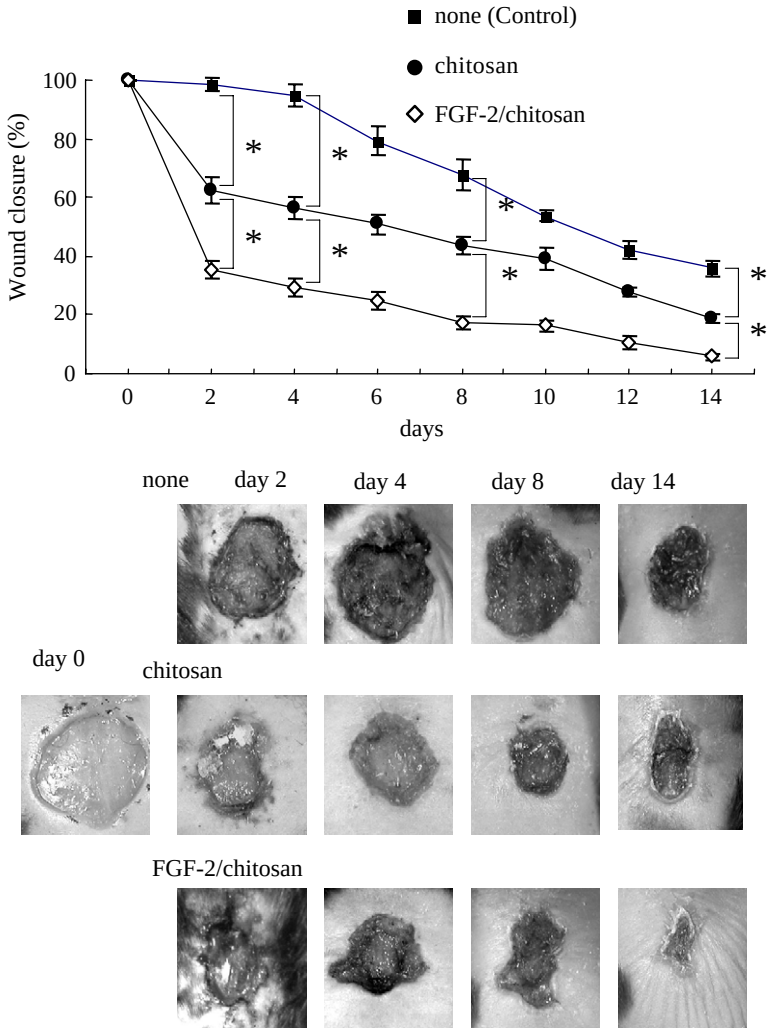


Fig. 4. Wound closure of FGF-2-incorporated chitosan hydrogel-treated *db*/*db* mice. In the upper panel, open wound areas of FGF-2-incorporated chitosan hydrogel-treated, chitosan hydrogel-treated, and control (none) wounds were determined every 2 day after initial wounding. The data represent the mean ± SE of eight mice. The statistical significance of wound closure was evaluated on post-wounding day 2, 4, 8, and 14. *Student *t* test, $p < 0.001$, $n = 8$. The lower panel represents photographic findings of wound repair covered with FGF-2-incorporated chitosan hydrogel, chitosan hydrogel, and control. The photographs of the wound at day 2, day 4, day 8, or day 14 are representative of eight mice in each group.

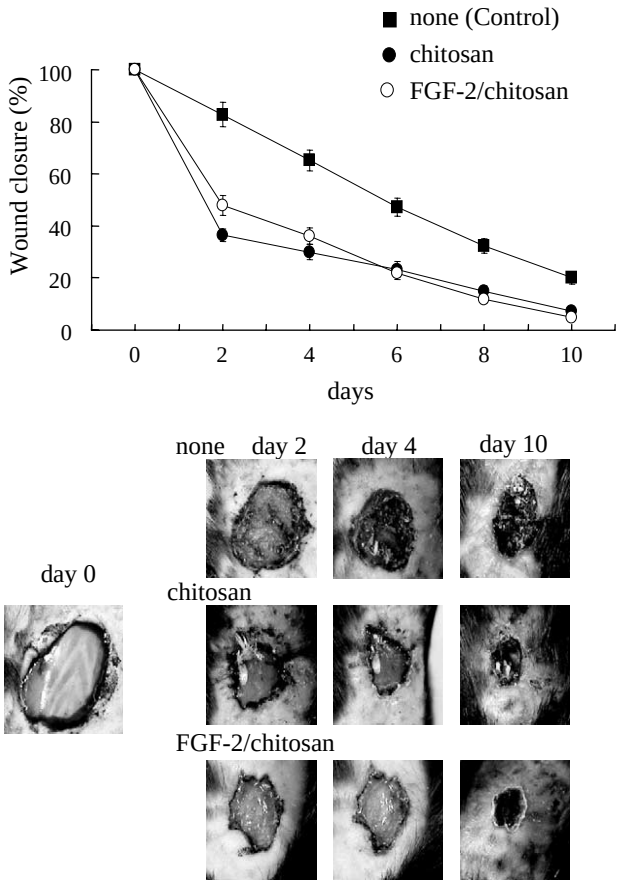


Fig. 5. Wound closure of FGF-2-incorporated chitosan hydrogel-treated *db*/+ mice. In the upper panel, open wound areas of FGF-2-incorporated chitosan hydrogel-treated, chitosan hydrogel-treated, and control (none) wounds were determined every 2 day after initial wounding. The wound closures of both chitosan hydrogel-treated and FGF-2-incorporated chitosan hydrogel-treated wounds on day 2, 4, 6, 8, and 10 significantly (Student t test, $p<0.001$, $n=8$) enhanced versus the control (none). However, there was no significant difference between the chitosan hydrogel-treated wounds and FGF-2-incorporated chitosan hydrogel-treated wounds. The data represent the mean$\pm$SE of eight mice. The lower panel represents photographic findings of wound repair covered with FGF-2-incorporated chitosan hydrogel, chitosan hydrogel, and control (none). The photographs of the wound at day 2, 4, and 8 are representative of eight mice in each group.

approximately 40% and 60% wound closure, respectively (Fig. 4). It is speculated that physical forces causing a wound to contract is initiated by adsorption of various protein molecules from the wound surface into the chitosan hydrogel. In fact, a hydrogel on a collagen sponge moistened with fetal bovine serum contracted to about 60% of its original volume in 2 days (data not shown).

On the other hand, wound healing in both chitosan hydrogel-treated and FGF-2 chitosan hydrogel-treated *db*/+mice occurred faster than the control wound and about 80% wound closure was achieved within 8 days. However, the incorporated FGF-2 in the chitosan hydrogel did not show a stimulatory effect on the wound healing of *db*/+mice (Fig. 5).

3.5. Histological observations on wound repair of db/db mice

A significant wound contraction was observed in both the chitosan hydrogel-treated and FGF-2-incorporated chitosan hydrogel-treated wounds of *db*/*db* mice in the first 2 days in contrast with the control (Fig. 6). Furthermore, granulation tissue formation and epithelialization in FGF-2-incorporated chitosan hydrogel-treated wounds progressed faster than in the control and chitosan hydrogel-treated wounds at day 4 (Fig. 7). The FGF-2-incorporated chitosan hydrogel-treated wounds were completely epithelialized and almost all necrotic tissues were replaced by new granulation tissue post-wounding on day 16. It should be noted that the wounds were well protected and firmly covered with the FGF-2-incorporated chitosan hydrogel in the first 10 days, and that the resulting hydrogel on the wound spontaneously fell off after day 16, and is speculated to be the result of the progress of epithelium formation.

4. Discussion

We have previously reported of a photocrosslinkable chitosan hydrogel (Az-CH-LA) containing both lactose moieties and photoreactive azide groups [8]. UV irradiation of an Az-CH-LA aqueous solution resulted within 10 s in an insoluble hydrogel, comparable to a soft rubber. It has been shown that the application of the chitosan hydrogel into open wounds induces a significant wound contraction, thereby accelerating the wound closure and healing process, as shown in a normal mouse model [10,11]. In addition, the chitosan hydrogel showed the ability of controlled release of various growth factors serving as a novel carrier and inducing neovascularization in vivo [14]. In the present work, we evaluated the effect of a FGF-2-incorporated chitosan hydrogel on the wound healing process using healing impaired *db*/*db* mice. Our main conclusions in this work are: (i) FGF-2 molecules incorporated into the chitosan hydrogel gradually release upon biodegradation of the hydrogel itself and (ii) FGF-2-incorporated chitosan hydrogels show a substantial effect to improve wound healing in the *db*/*db* mice.

Several studies have shown that chitin and chitosan accelerate wound healing and remedies using chitin and chitosan in wound healing treatments have already been on the market [15,16]. However, in most studies the chitin and chitosan have been used as filament, powder, granule, sponge or composite with cotton or polyester [17,18]. The moist healing environment and accelerating effect of the chitin and chitosan could therefore not be

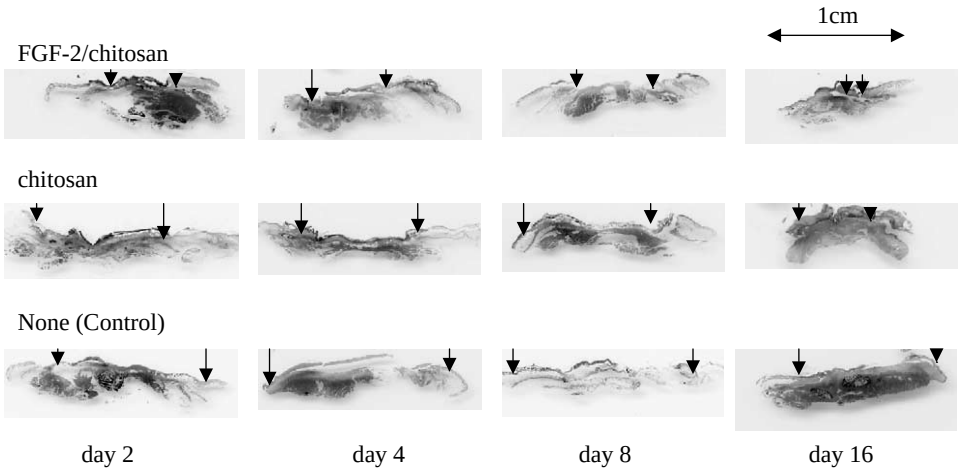
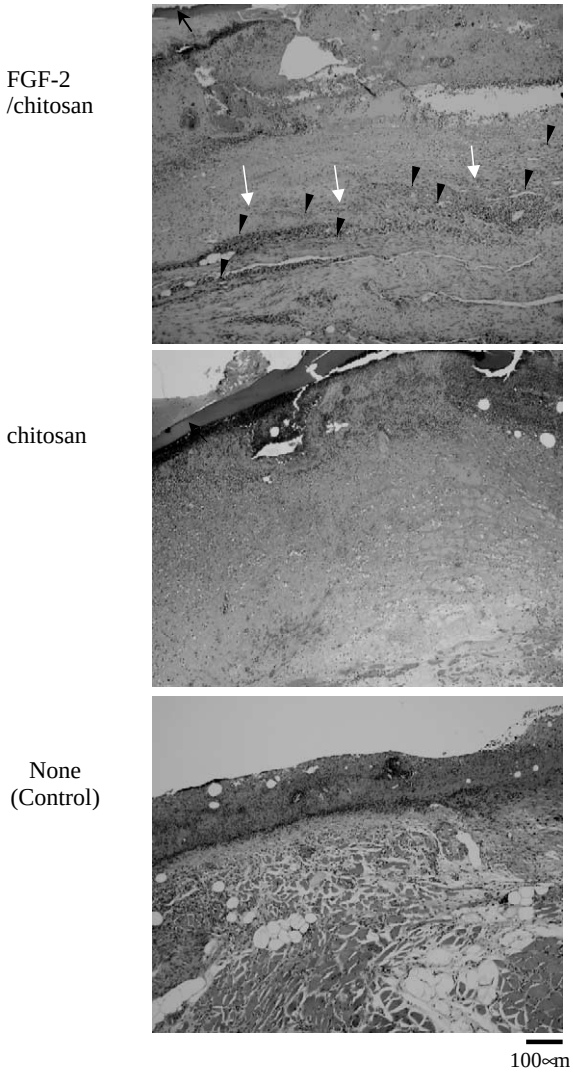


Fig. 6. Histological examination of wound repair of FGF-2-incorporated chitosan hydrogel-treated, chitosan hydrogel-treated, and control (none) wounds of *db*/*db* mice on day 2, 4, 8, and 16 after initial wounding. The photographs on each day are representative of eight wounds stained with hematoxylin & eosin (H&E) in each group. Arrows show edges of the epithelialization of each wound.



exploited to its maximum due to the relatively low interaction between the wound and these healing agents. However, application of a chitosan hydrogel as an occlusive dressing is able to effectively interact with, protect and contract the wound, in a suitable moist healing environment.

Besides FGF-2, other polypeptide growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) have been shown to enhance various parameters of wound healing [19–22]. Mutant diabetic (*db*/*db*) mice have elevated blood sugar levels, increased or normal insulin concentrations and a suppressed cell-mediated immunity [12]. These mice are also obese with a distended, thin skin. Thus, the reason for their impaired rate of wound healing may be complicated and multifaceted. It is known that glucocorticoid-treated rats have delayed wound healing and that TGF-β is able to reverse this effect [23]. Rats with chemically induced diabetes show a decreased rate of collagen deposition, which can be restored to a normal level by an application of PDGF [20]. Although the mechanism responsible for impaired wound healing in *db*/*db* mice is not completely understood, it is likely that the presence of macrophages has a significant effect on

Fig. 7. Histological examination of wound repair of FGF-2-incorporated chitosan hydrogel-treated, chitosan hydrogel-treated, and control (none) wounds of *db*/*db* mice on day 4 after initial wounding. The photographs are representative of eight wounds stained hematoxyline & eosin (H&E) in each group. White arrows in the FGF-2-incorporated chitosan hydrogel-treated wound show edges of the granulation formation and black triangles show vascularization sites. Any granulation and vascularization formations were not observed in the control wound (none) and the chitosan hydrogel (chitosan)-treated wound on day 4. The black arrow shows remaining chitosan hydrogel in the wound (original magnification $\times$ 100).

the formation of wound granulation tissue [24], and that macrophage accumulation is impaired in *db*/*db* mice. Furthermore, a defect in vascular endothelial growth factor (VEGF) expression is suggested to be associated with a wound-healing disorder [25].

When a FGF-2 solution was applied once a day to an open wound of *db*/*db* mice, FGF-2 showed a significant effect on granulation formation, infiltrating cells, and capillary number, but showed only a minor effect on the degree of re-epithelialization [2]. Probably the presence of a large scab formed in the open wound system, negatively affected the migration of keratinocytes. In the present study, the application of a chitosan hydrogel into the open wound did not form large scabs, and FGF-2 in the chitosan hydrogel obviously enhanced re-epithelialization, as well as other parameters in the wound healing process. It is also interesting that a minor effect of FGF-2 in the chitosan hydrogel on the degree of healing in normal (*db*/+) mice was observed. It is possible that *db*/+mice have a sufficient amount of growth factors to achieve a maximal rate of healing, and thus only a minor increase in wound repair is possible upon application of exogenous FGF-2. The wound closure assay and histological examination used in this study may not be sensitive enough to detect these small effects. These results suggest that the FGF-2-incorporated chitosan hydrogel may be a promising wound dressing, especially in the treatment of healing-impaired wounds.

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